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COMMENTARY

Attractive and repulsive factors act through multi-subunit receptor complexes to regulate nerve fiber growth

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ABSTRACT

In the nervous system, attractive and repulsive factors guide neuronal growth, pathfinding and target innervation during development, learning and regeneration after injury. Repulsive and growth-inhibitory factors, such as some ephrins, semaphorins, netrins and myelin-associated growth inhibitors, restrict nerve fiber growth, whereas neurotrophins, and other ephrins, semaphorins and netrins attract fibers and promote neurite growth. Several of these guidance molecules also play crucial roles in vasculogenesis, and regulate cell migration and tissue formation in different organs. Precise and highly specific signal transduction in space and time is required in all these cases, which primarily depends on the presence and function of specific receptors. Interestingly, many of these ligands act through multi-subunit receptor complexes. In this Commentary, we review the current knowledge of how complexes of the receptors for attractive and repulsive neurite growth regulatory factors are reorganized in a spatial and temporal manner, and reveal the implications that such dynamics have on the signaling events that coordinate neurite fiber growth.

KEY WORDS: Ephrin, Functional microdomain, Myelin-associated inhibitors, Neurotrophins, Receptor complex

Introduction

The outgrowth of axons and pathfinding to their targets is accurately guided by environmental cues in the developing central and peripheral nervous system. The modulation of the neuronal growth cones by growth-promoting or -restricting neurotrophic and axonal guidance factors is not only relevant for axonal patterning during development, but also for plastic circuit rearrangements and fiber regeneration following injury. Interestingly, many of these ligands are also present at synapses in the adult nervous system, where they influence synaptic plasticity and learning. Axon guidance molecules, such as ephrins, netrins, as well as neurotrophins and myelin-associated growth inhibitory molecules, signal through dynamically assembled multi-subunit receptor complexes, which comprise several, often structurally unrelated binding partners (Fig. 1). Furthermore, semaphorin (SEMA)-family members, acting as repulsive or growth-promoting cues, signal through plexins (PLXNs) and a diversity of co-receptors. SEMA3 proteins additionally require neuropilins (NRP1 or NRP2) for repulsive neuronal signaling. Semaphorin-mediated axon pathfinding is of particular importance during development of the early spinal cord and the retinothalamic tract (Kuwajima et al., 2012; Pasterkamp, 2012; Yoshida, 2012). In this Commentary, we will first introduce the main interaction partners of neurotrophins, ephrins, netrins and myelin-associated inhibitors that are relevant in this context, as well as their effects on nerve fiber growth. We will then review and compare the events during formation of the early receptor complex, such as the recruitment and assembly of receptors and co-receptors, before discussing studies of ligand-induced membrane mobility and complex internalization, and emphasizing their implications in downstream signaling events.

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Neurotrophic and axonal guidance factors, and their receptors

Neurotrophins

The family of neurotrophins comprises nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3, also known as NTF3) and neurotrophin-4 (NT4, also known as NTF4 and neurotrophin-5) (Chao, 2003; Chao et al., 2006). All neurotrophin-family members bind to high-affinity tropomyosin receptor kinases (Trks); however, they bind with different preferences – NGF binds preferentially to TrkA, BDNF and NT4 to TrkB, and NT3 to TrkC (TrkA, TrkB and TrkC are also known as NTRK1, NTRK2 and NTRK3, respectively) (Barbacid, 1995; Ip et al., 1992). The low affinity tumor necrosis factor (TNF)-receptor-family member p75 (TNFRSF1B) interacts with all neurotrophins, both in the presence and absence of Trks as co-receptors (Hempstead et al., 1991). Neurotrophin signaling through Trks and p75 promotes neurite outgrowth, differentiation and cell survival (Huang and Reichardt, 2001), whereas the precursor peptide pro-NGF selectively induces cell death through p75 and the neurotensin receptor sortilin in the absence of TrkA (Jansen et al., 2007; Kotlyanskaya et al., 2013; Lee et al., 2001; Nykjaer et al., 2004; Teng and Hempstead, 2004). NGF is synthesized and secreted by sympathetic and sensory target cells to attract and stabilize the corresponding axons (Huang and Reichardt, 2001; Korsching, 1993). After peripheral nerve injury, NGF is released by Schwann cells, fibroblasts and infiltrating mast cells, and is important for the survival and regeneration of injured neurons (Huang and Reichardt, 2001). In the central nervous system (CNS), the basal forebrain and striatal cholinergic neurons in particular respond to NGF, whereas cerebellar granule cells, mesencephalic dopaminergic neurons, hippocampal neurons and retinal ganglion cells respond to BDNF and NT4 (Huang and Reichardt, 2001).

Netrins

The netrin-family members are chemotropic factors, acting either as attractive or repulsive ligands, depending on the concentration and the receptor composition on the responding cell. Netrin-1, netrin-2 and netrin-3 are secreted ligands, which signal through the receptors deleted colorectal cancer (DCC), the DCC paralog neogenin, the UNC-5 homologs UNC5A–D, and Down syndrome cell adhesion molecule (DSCAM) (Lai Wing Sun et al., 2011; Xu et al., 2014). Glycosylphosphatidylinositol (GPI)-linked netrins G1 and G2 interact with netrin-G ligands (NGLs) NGL-1 (also known as LRRC4C) and NGL-2 (also known as LRRC4) (Lai Wing Sun et al., 2011; Yurchenco and Wadsworth, 2004). Netrin-1 is

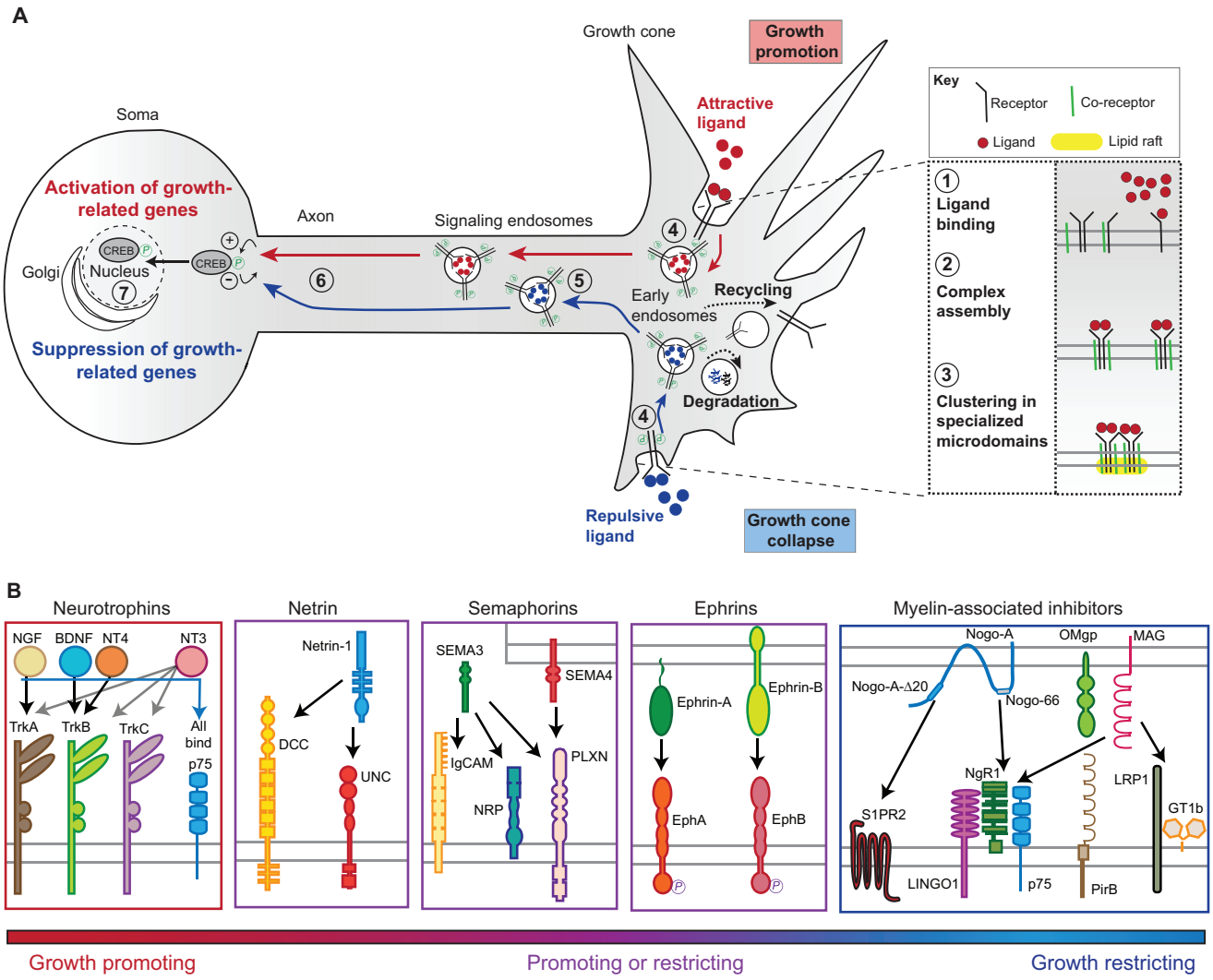

Fig. 1. Cellular pathways coordinating neuronal attraction and repulsion at the ligand–receptor level. (A) Attractive or repulsive ligands that regulate nerve fiber growth bind to their high-affinity binding partners at the neuronal growth cone (1). Subsequently, co-receptors and additional complex partners are recruited (2). The binding partners often multimerize in the presence of the ligand and translocate to specialized microdomains, which stabilize the initial interaction (3). For several of the factors that regulate nerve fiber growth, ligand–receptor complexes are internalized and trafficked intracellularly (4). Early endosomes act as early sorting stations, defining the subsequent trafficking routes. Some complex partners undergo degradation or recycling back to the membrane (5). Distinct ligand–receptor complexes are transported in a retrograde manner within signaling endosomes from the neuronal growth cone to the cell body (6). Here, through their downstream-signaling cascades, they induce the activation or deactivation of transcription factors (by phosphorylation or dephosphorylation), which in turn controls the expression of growth-promoting or -restricting genes in the nucleus (7). (B) Scheme of the attractive and repulsive cues, and their main receptors as discussed in this review. BDNF, brain-derived neurotrophic factor; DCC, deleted colorectal cancer; Eph, Ephrin receptor; GT1b, a ganglioside; IgCAM, immunoglobulin superfamily cell adhesion molecules; LRP1, lipoprotein receptor related protein; MAG, myelin-associated glycoprotein; NGF, neurotrophic growth factor; NgR1, Nogo receptor; NRP; neuropilin; NT3, neurotrophin-3; NT4, neurotrophin-4 (also known as neurotrophin-5); OMgp; oligodendrocyte myelin glycoprotein; p75, low-affinity nerve growth factor receptor (also known as TNFRSF1B); PirB, paired immunoglobulin-like receptor B; PLXN, plexin; S1PR2, sphingosine-1-phosphate receptor 2; SEMA, semaphorin; Trk, tropomyosin receptor kinases A–C, UNC; uncoordinated (vertebrate UNC-family members UNC5A–D, Netrin receptors).

expressed in various regions of the CNS during development and adulthood (Lai Wing Sun et al., 2011). During neuronal development, netrins guide cell and axon migration, and influence axonal arborization and synapse formation. Netrin-positive cells in the midline of the early neural tube have a crucial role in attracting migrating DCC-expressing neuronal precursor cells in the developing hindbrain (Alcantara et al., 2000; Lai Wing Sun et al., 2011). In the developing spinal cord, a netrin-1 gradient that is generated by floor-plate cells attracts the commissural axons. The gradient also repels migrating oligodendrocyte precursor cells (OPCs) and the axons of trochlear motoneurons in the brainstem (Jarjour et al., 2008, 2003; Lai Wing Sun et al., 2011). In the adult CNS, netrin-1 influences cell–cell contacts, e.g. paranodal junctions of oligodendrocytes. Beyond the crucial role of netrin signaling in the CNS, a number of diverse cell biology processes outside of the CNS are affected by netrins, such as angiogenesis and the development of mammary glands, lungs and pancreas (Cirulli and Yebra, 2007; Jarjour et al., 2008; Lai Wing Sun et al., 2011).

Ephrins

Interactions between ephrins and ephrin receptors (Ephs) guide axons during development, and during recovery processes after injury. There are two classes of ephrin ligands – ephrin-A ligands (of which there are five, ephrin-A1–A5), which are linked to GPI,

and transmembrane ephrin-B ligands (of which there are three, ephrin-B1–B3). Ephrin-A ligands interact predominantly with one of the eight EphA receptors (EphA1–A8), whereas ephrin-B ligands interact primarily with one of the four EphB receptors (EphB1–B4) (Grunwald and Klein, 2002). Interestingly, interactions between ephrin ligands and Eph receptors could lead to bidirectional signaling – ephrins on one cell can activate Eph receptors on a second cell (forward signaling), or Eph on the second cell can act as a ligand and activate ephrins, which function as receptors, on the first cell (reverse signaling) (Pasquale, 2005, 2008; Steinecke et al., 2014). During the development of spinal-motoneuron-mediated muscle innervation, Eph–ephrin signaling has an important role in the correct pathfinding and target innervation by repelling outgrowing axons from areas with high ephrin levels, and by promoting outgrowth into regions that express low levels of ephrins (Klein and Kania, 2014; Pasquale, 2005). Retinal ganglion axons are guided towards the superior colliculus and optic tectum area, which is one of their main targets, by ephrin-A and EphA gradients. The expression of EphA3 in the retinal ganglion cells increases along the nasal–temporal axis. In the optic tectum, the expression of ephrin-A2 and ephrin-A5 forms a gradient with low levels at anterior regions and high levels at posterior regions. EphA3-mediated repulsive (forward) signaling therefore prevents temporal retinal ganglion cell axons from growing into the posterior tectal regions that express high levels of ephrin-A2 and ephrin-A5. Nasal retinal ganglion cell axons that express low EphA3 levels are able to grow into the posterior optic tectum because they experience a lower level of repulsion (Klein and Kania, 2014; Pasquale, 2005; Triplett and Feldheim, 2012). Ephrins and Ephs also crucially influence the correct formation of the corticospinal tract. Ephrin-B3 ligands, which are expressed at the midline of the brainstem and the spinal cord, interact with EphA4 receptors on corticospinal axons, and so prevent a re-crossing of the corticospinal fibers and ensure proper cortical projection into the spinal cord (Dottori et al., 1998; Grunwald and Klein, 2002; Klein and Kania, 2014). Ephrin-A5- and ephrin-B1-mediated signaling in early embryonic cortical precursors, as well as in sympathetic neurons, has been shown to promote neurite outgrowth and survival; however, the underlying modes of action still need to be defined (Gao et al., 2000; Zhou et al., 2001). Heparan sulfate has been identified as a modulator of ephrin-mediated signaling because ephrin-A3-dependent rounding of Chinese hamster ovary (CHO) cells and collapse of neuronal growth cones depends on heparan sulfate (Irie et al., 2008). Besides the crucial role of ephrin and Eph signaling in the nervous system, these ligand–receptor pairs have been shown to also modify many aspects of cancer development and progression, such as the growth, migration and invasion of cancer cells (Pasquale, 2008, 2010; Surawska et al., 2004).

Myelin-associated neurite growth inhibitors

CNS myelin, but not peripheral nervous system (PNS) myelin inhibits axon outgrowth (Caroni and Schwab, 1988; Schwab and Thoenen, 1985). Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp, also known as OMG) are myelin-associated inhibitors of neurite outgrowth (Filbin, 2003; Schwab, 2010). Nogo-A (also known as Rtn4a in zebrafish) is a member of the reticulon (Rtn)-family of membrane proteins; it has two main extracellular inhibitory domains, Nogo-66 and Nogo-A-Δ20. These domains interact with different receptor complexes, the downstream signals of which result in the activation of RhoA and Rho kinase (ROCK, for which there are two isoforms ROCK1 and ROCK2), the destabilization of the cytoskeleton and a transcription-dependent downregulation of the neuronal growth program (Kempf and Schwab, 2013; Schwab, 2010; Schwab and Strittmatter, 2014). Nogo-A-Δ20 elicits the inhibition of neurite outgrowth and fibroblast spreading through the G-protein-coupled receptor sphingosine-1-phosphate receptor 2 (S1PR2) (Kempf et al., 2014). Interestingly, Nogo-A-Δ20 also binds to the membrane protein tetraspanin-3 (TSPAN-3), and the Nogo-A–TSPAN-3 complex then co-clusters with S1PR2. Accordingly, Nogo-A-induced growth inhibitory effects and RhoA activation are reduced when TSPAN-3 is depleted from the responding cells (N.K.T.-S., Björn Tews, David Albrecht, Zorica Ristic, Helge Ewers and M.E.S., unpublished observation). The second active domain of Nogo-A, Nogo-66, binds to the Nogo receptor 1 (NgR1, also known as RTN4RL1) (Fournier et al., 2001), which acts in concert with the co-receptors p75 or TROY (TNFRSF19), and LINGO1 (Mi et al., 2004; Park et al., 2005; Shao et al., 2005; Wang et al., 2002). Nogo-66 also binds to the paired immunoglobulin-like receptor B (PirB, also known as PILRB) (Atwal et al., 2008; Schwab, 2010).

Interestingly, the two other myelin-associated inhibitors MAG and OMgp also bind to NgR1, in complex with the same co-receptors – i.e. p75 or TROY, and LINGO1 – although MAG shows a higher binding affinity for the Nogo-66 receptor homolog NgR2 (RTN4RL2) (Giger et al., 2010; Schwab, 2010). MAG further interacts with gangliosides, which are involved in inhibition of MAG-induced neurite outgrowth (Vinson et al., 2001). These myelin-associated inhibitors are predominantly thought to act as inhibitors of regeneration, circuit plasticity and functional recovery after injury to the CNS (Filbin, 2003; Schwab, 2004; Yiu and He, 2006). In addition to restricting axonal regeneration and structural plasticity after spinal cord injury or stroke (Schwab and Strittmatter, 2014; Wahl et al., 2014), Nogo-A, NgR1 and PirB have crucial roles in restricting plasticity in the visual system, the sensory-motor cortex and the hippocampus in the uninjured adult CNS (Akbik et al., 2012; Bochner et al., 2014; Delekate et al., 2011; McGee et al., 2005; Schwab, 2010; Schwab and Strittmatter, 2014; Zemmar et al., 2014). Through its effects on cell adhesion and cytoskeletal dynamics, Nogo-A-Δ20 influences the migration of neuronal precursors (through NgR1) and inhibits the migration of primary brain microvascular endothelial cells (through S1PR2), as well as the spreading of endothelial cells and fibroblasts (Schwab, 2010; Walchli et al., 2013). This indicates that specific ligand-induced signaling pathways and effects, such as the destabilization of the cytoskeleton, are conserved in different cell types, resulting in related cellular effects, such as neuronal growth cone collapse, inhibition of migration, or spreading of fibroblasts or neuronal precursors.

Dynamic receptor complex formation at the cell membrane

Neuronal growth-promoting or -restricting molecules signal in the developing and mature CNS through receptor complexes that comprise different interaction partners. The concerted interplay between signal-transducing receptors, their modulatory co-receptors and accessory factors is initiated by their ligands, although some binding partners can co-associate even in the absence of the ligand in signaling-incompetent complexes. These ligand-induced receptor complexes assemble in a dynamic manner into complexes that range in size from nano- to microscale and undergo structural reorganizations, giving rise to a functional signaling platform as discussed below (Fig. 2).

Initial events upon ligand binding – recruitment, assembly and multimerization of binding partners

Neurotrophins were one of the first examples of ligands of multi-subunit receptor complexes to be studied in detail (Chao, 2003;

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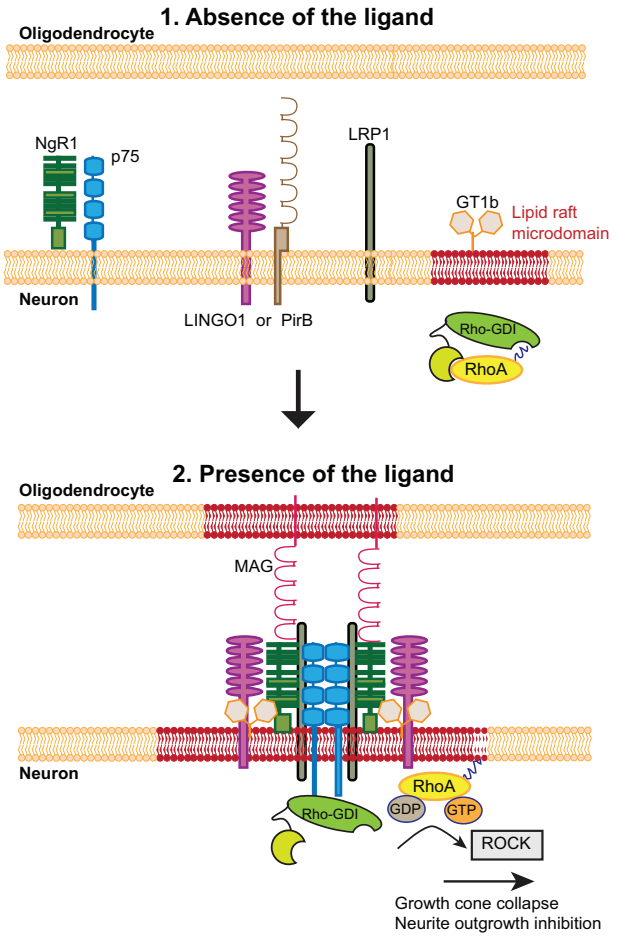

Fig. 2. Ligand-induced formation of receptor complexes at the cell membrane. Illustrated here is the formation of the multi-subunit heteromeric receptor complex of myelin-associated glycoprotein (MAG) as an example of a growth-restricting ligand. In the absence of the ligand, the binding partners of MAG do not form a fully functional complex, although the Nogo receptor (NgR1) and p75 (also known as TNFRSF1B) partially colocalize (shown on the top). In the presence of MAG, the receptor components assemble into a signal-transducing complex, which is stabilized by its translocation into lipid rafts (shown at the bottom). MAG presentation increases the association between NgR1 and p75 (or PirB) and mediates the recruitment of LRP1 into the vicinity of p75. Gangliosides such as GT1b stabilize the interaction of NgR1 with the co-receptor LINGO1. Lipid rafts scaffold the MAG receptor complex and facilitate downstream signaling, for instance p75-mediated release of Rho GDP-dissociation inhibitors (Rho-GDI) from the GTPase RhoA. Subsequent activation of RhoA and Rho kinase (ROCK, for which there are two isoforms ROCK1 and ROCK2) results in destabilization of the cytoskeleton, followed by collapse of the growth cone and inhibition of neurite outgrowth.

Teng and Hempstead, 2004). Neurotrophins induce dimerization of Trks, although it has been shown recently that inactive Trk homodimers and also p75–TrkA heterodimers exist in the absence of ligands (Iacaruso et al., 2011; Jing et al., 1992; Shen and Maruyama, 2011). Crystal structure analysis and biochemical experiments indicate a 2:2 stoichiometric binding of NGF or NT3 to p75 in solution (Aurikko et al., 2005; Gong et al., 2008). Thus, the presence of dimerized NGF or NT3 promotes homodimerization of p75, dimerization and activation of Trk, as well as formation of heterotrimeric receptor complexes containing Trk proteins and p75.

Furthermore, netrin-family members act as guidance molecules, interacting with different partners of the receptor complex and activating attractive- or repulsive-signaling pathways dependent upon the respective interaction partners. Netrin-1 signaling through DCC induces chemoattraction, but signaling through UNC-5 proteins induces chemorepulsion (Lai Wing Sun et al., 2011). It has recently been shown that neogenin, which is structurally similar to DCC, acts as an additional receptor of netrin-1 that, in collaboration with DCC, mediates the attraction of commissural neurons in the developing spinal cord. The binding of netrin-1 to either DCC or neogenin results in a differential architecture of the ligand–receptor complex – a 2:2 heterotetramer for the netrin-1–neogenin complex and a continuous chain-like ligand–receptor complex in the case of netrin-1 and DCC. These distinct architectures of the ligand–receptor complexes might provide the molecular basis for the different signaling events that they elicit (Xu et al., 2014). The structural insight into the binding of netrin-1 to two DCC molecules is in line with a second crystallography study that has defined two binding sites on netrin-1 – one that is specific for DCC and a second that could interact either with a second DCC molecule or UNC5. The choice between netrin-1 binding to either DCC or UNC5 with its second binding site is governed by the binding of netrin-1 to one of two distinct heparan sulfate molecules (HP1 and HP2) – when netrin is bound to either HP1 or HP2, the second site is selective for DCC or UNC5, respectively. Netrin-1–DCC signaling induces attraction of the growth cone, whereas netrin-1–UNC5 induces growth cone repulsion (Finci et al., 2014). Furthermore, netrin-1 interacts with other binding partners, such as the laminin-binding integrins α6β4 and α3β1, and this has been implicated in the activation of adhesion and migration of pancreatic epithelial cells, which express netrin-1 (Lai Wing Sun et al., 2011; Ly et al., 2008; Moore et al., 2007; Yebra et al., 2003). These different interactions of netrins with their partners influence the cell biology aspects of different cell types in either an attractive or repulsive manner.

Nogo-A also interacts with multiple binding partners. The GPI-anchored Nogo-66 receptor NgR1 is important for binding of Nogo-66, MAG or OMgp, but because it lacks a signal-transducing cytoplasmic domain, it depends on the co-receptors p75 and TROY to form a signal-transducing complex (Wang et al., 2002). LINGO1, another component of the Nogo receptor complex, forms stable tetramers, which appear to be necessary for its interaction with the receptor-complex partners NgR1 and p75 (Mosyak et al., 2006). LINGO1 and the ectodomain of NgR1 are characterized by evolutionary conserved leucine-rich repeat domains (Filbin, 2003; Saha et al., 2014). NgR1 and p75 colocalize even in absence of their myelin-associated inhibitory ligands; however, presence of the ligand increases the association between NgR1 and p75 in a concentration-dependent manner (Wang et al., 2002; Wong et al., 2002). In addition to NgR1, MAG also binds to the lipoprotein-receptor-related protein LRP1. This binding does not depend on the presence of p75, but induces a colocalization of LRP1 and p75, which is not detectable in absence of the ligand (Mantuano et al., 2013; Stiles et al., 2013). This dynamic recruitment of p75 to MAG and/or LRP1 is followed by the activation of RhoA (Fig. 2). An active recruitment of p75 into MAG-bound receptor complexes has been also shown for PirB, another receptor for Nogo-66 and MAG (Fujita et al., 2011; Shao et al., 2005). The interaction partners of Nogo-66 and MAG assemble into a functional complex, comprising a high-affinity binding partner (NgR1), signal-transducing receptors (p75 and TROY) and a complex-stabilizing co-receptor (LINGO1), which cooperatively transduces the ligand-induced inhibitory effects.

Once the initial ligand–receptor pair assembles into a small functional entity, multimers of the receptor and co-receptors can be recruited, often in a specific stoichiometry. Ligand-induced

clustering and oligomerization of receptors and co-receptors culminates in an enhanced signaling output of the resulting receptor cluster, as observed for ephrins, myelin-associated growth inhibitors, SEMA3A and neurotrophins (Barton et al., 2004; Gong et al., 2008; Himanen et al., 2010; Janes et al., 2012; Janssen et al., 2012; Marchetti et al., 2013; Mosyak et al., 2006; Pasquale, 2005; Salaita et al., 2010; Seiradake et al., 2010; Wimmer-Kleikamp et al., 2004). In this context, the number of ligand–receptor pairs in these clusters directly correlates with the strength and outcome of the elicited response (Janssen et al., 2012, 2010; Schaupp et al., 2014). For instance, the multimer composition of EphB2 clusters (monomers, dimers, tetramers) determines the strength of the cellular repulsion response – the more EphB2 receptor dimers that are present, the weaker the response, and the more tetramers there are, the stronger the response (Schaupp et al., 2014). Although EphA and EphB subtypes preferentially bind to A- and B-type ephrins, respectively, mixed oligomers comprising EphA and EphB exist on cells that express both Eph subtypes, and these oligomers modulate signaling and cell retraction (Janes et al., 2011). Distinct patterns of EphA and EphB hetero-oligomerization exist on breast, prostate and glioblastoma tumor cells, and their modulatory signaling might influence physiological Eph–ephrin signaling, thereby promoting cancer progression (Al-Ejeh et al., 2014). The assembly of ephrin–Eph complexes into higher-order signaling clusters is mediated by the initial dimerization of the cysteine-rich domain of Ephs; at high concentrations, this might lead to receptor clustering even in absence of the ligand (Himanen et al., 2010). The multivalent binding of a ligand (simultaneous binding to one assembly of receptors) has recently been shown to drive receptor clustering at the nanoscale level (Conway et al., 2013). Super-resolution microscopy has been used to visualize ephrin-B2-driven clustering of the EphB4 receptor at the nanoscale level on adult neuronal stem cells, as ephrin-B–EphB signaling has been shown to regulate neuronal stem cell differentiation (Ashton et al., 2012; Conway et al., 2013; Vazin et al., 2009). The multivalency of a recombinant ephrin-B2 ectodomain conjugated to a soluble biopolymer strongly enhanced the bioactivity of ligands, resulting in the induction of neuronal stem cell signaling – i.e. β-catenin expression – and stem cell differentiation (Conway et al., 2013). Other studies have shown the formation of large EphA3-signaling clusters that are induced by the multivalent ligand ephrin-A5. In those studies, the size of the EphA3 clusters exceeded that of the initial ephrin-A5–EphA3 contact site by several fold (Janes et al., 2012; Wimmer-Kleikamp et al., 2004). The expansion of signaling clusters from 'seed' ligand–receptor interactions leads to the recruitment of additional Ephs in an ephrin-independent manner. This has been described as the so-called 'nucleation' mechanism, and crystal structure analyses provide further evidence for it (Seiradake et al., 2010; Wimmer-Kleikamp et al., 2004). The nucleation mechanism supports the notion of signal propagation and an increase in sensitivity to the ephrin-ligands.

A single ligand can also drive distinct cellular responses depending on the receptor it interacts with (Marchetti et al., 2013; Seiradake et al., 2013). For instance, structure-based differences between EphA4 and EphA2 lead to differential binding of their ligand ephrin-A5. Presentation of ephrin-A5 to HeLa cells transfected with either EphA4 or EphA2 results in opposing effects – i.e. in either collapse or adherence of the cell, which are associated with smaller clusters of ephrin-A5–EphA4 and larger clusters of ephrin-A5–EphA2, respectively. Therefore, a relatively small number of distinct receptors are able to control diverse signaling pathways (Seiradake et al., 2013). Taken together, these studies indicate that the initial binding of different attractive and repulsive ligands recruits additional co-receptor and partner molecules into a signaling-competent complex. Further fine-tuning can occur through modulation of the specific complex composition and the stoichiometry of the signal-transducing components present therein.

Specialized microdomains as signaling platforms

The presence of a ligand induces the dynamic assembly of multiple interaction partners into signaling scaffolds. These scaffolds concentrate the specific subunits of the receptor complex into distinct areas or microdomains at the plasma membrane, and potentially amplify the transduction of the elicited signal. Specific plasma membrane constituents facilitate ligand-induced assembly of the receptor complex. In this context, lipid rafts are of particular importance. They comprise dynamic assemblies of cholesterol and sphingolipids, and are enriched in GPI-anchored proteins, cholesterol- or palmitoyl-linked proteins and the α-subunits of heterotrimeric G-proteins (Simons and Toomre, 2000). In neurons, the interaction between MAG and its receptors – NgR, and the GT1b and GD1 gangliosides – occurs in lipid rafts. Therefore, it has been suggested that these rafts form discrete areas that support the high-avidity multivalent binding of MAG to its interaction partners, and that they contain the MAG receptors and the downstream effector RhoA (Vinson et al., 2003) (Fig. 2). Similar results, revealing the relevance of lipid rafts, have been described for the interaction of Nogo-66 with its receptors p75 and NgR1; in this case, the receptors colocalize in lipid rafts, and Nogo-66 signaling is disturbed in the presence of the cholesterol-depleting substance β-methylcyclodextrin (Yu et al., 2004). Gangliosides, sialic-acid-bearing glycosphingolipids, are required for MAG-induced translocation of p75 to lipid rafts and subsequent signal transduction (Fujitani et al., 2005). Furthermore, gangliosides mediate the interaction between NgR1 and its co-receptor LINGO1 (Saha et al., 2011), and it has been suggested that the binding of MAG to gangliosides influences the optimal axon–myelin interaction (Schnaar, 2010) (Fig. 2).

The effect of gangliosides on NGF–TrkA-signaling is not fully understood. Exogenous ganglioside GM1 enhances NGF-induced dimerization of TrkA and its phosphorylation (Farooqui et al., 1997; Mutoh et al., 1995), but endogenous GM1 suppresses NGF signaling, probably by changing the intracellular localization of NGF receptors (Nishio et al., 2004). Lipid rafts have been shown to positively affect neurotrophin signaling – binding of NGF to TrkA induces its translocation and concentration in lipid rafts, which facilitates the formation of receptor-associated signaling complexes, their internalization and the activation of NGF-induced downstream signaling, such as phosphorylation of TrkA and activation of the extracellular signal-regulated kinase (ERK) pathway (Limpert et al., 2007). Ligand-induced receptor translocation to lipid rafts has also been shown for BDNF–TrkB. In lipid rafts, TrkB-bound BDNF associates with the lipid-raft-resident protein Fyn, which mediates downstream signaling of BDNF in neurons (Pereira and Chao, 2007).

An important consequence of partitioning multi-subunit receptor complexes into lipid rafts upon ligand presentation could be the resulting selectivity of the signals transduced (Campbell et al., 2008; Zonta and Minichiello, 2013). An example of this is the distinct membrane compartmentalization and signaling of ephrin-A5 and ephrin-B1, as studied in NIH 3T3 fibroblasts, where ephrin-A5 is found in detergent-resistant membrane fractions, whereas ephrin-B1 translocates to these membrane fractions only upon receptor binding. In detergent-resistant membrane fractions, ephrin-A5 and ephrin-B1 fractionate at different raft fractions in the living cell. This results in differential regulation of the cytoskeletal architecture and

distinct gene expression, as assessed by using microarray analysis of NIH 3T3 fibroblasts that expressed ephrin-A5 or ephrin-B (Campbell et al., 2008; Gauthier and Robbins, 2003).

In conclusion, multimeric and multi-subunit receptor complexes that interact with and transduce attractive and repulsive axonal guidance cues frequently form in specialized plasma membrane microdomains, the lipid rafts. The high avidity of the multivalent ligand–receptor interactions facilitate, amplify and specify the resulting intracellular signals (Farooqui et al., 1997; Fujitani et al., 2005; Limpert et al., 2007; Pereira and Chao, 2007; Vinson et al., 2003; Yu et al., 2004; Zonta and Minichiello, 2013).

Translocation and internalization of receptor complexes

The ligand-induced formation of functional receptor complexes is accompanied by the lateral motion of the individual binding partners and receptor components in the membrane. Subsequent to their assembly, receptor complexes are often internalized into signaling endosomes that contain the endocytosed ligand–receptor complexes and downstream effectors. Below, we review the known patterns of movement of attractive and repulsive multi-subunit receptor complexes, and compare the changes associated with these both at the cell membrane and inside the cell.

Ligand-induced lateral receptor dynamics

Compelling evidence for the importance of ligand-induced lateral receptor reorganization has been provided for the EphA2–ephrin-A1 pair (among the Eph–ephrin interactions) (Greene et al., 2014; Salaita et al., 2010; Xu et al., 2011) (Fig. 3). In these studies, EphA2-expressing epithelial cells were cultured on a supported membrane, which included laterally mobile and fluorescently labeled ephrin-A1. At the interface between the supported membrane and the cell membrane, ephrin-A1 induced clustering and a spatial reorganization of EphA2 receptors that was associated with the recruitment of the membrane metalloproteinase ADAM10 and a rearrangement of the cytoskeleton. However, when the lateral mobility of ephrin-A1 was restricted by using nanofabricated physical barriers beneath the supported membranes, there was a drastic change in the cellular response – the spatial reorganization of EphA2, its phosphorylation at tyrosine residues, and the association of EphA2 clusters with the actin cytoskeleton and ADAM10 were all restricted to the pattern of the physical barrier (Fig. 3). Physical interference with the receptor reorganization also impaired the internalization of the receptor–ligand signaling complex (Greene et al., 2014; Salaita et al., 2010; Xu et al., 2011). On supported membranes, physical restriction of ephrin-A1 by using ordered arrays of gold nanodots was used to compare the lateral mobility of EphA2 in the membranes of different breast cancer cells (Lohmüller et al., 2013). This revealed that the cancer cell lines with the highest tumorigenicity and metastatic potential exhibited the most restricted relocation of EphA2, as assessed by using nanodot arrays. Thus, strong EphA2 clustering and an altered EphA2–ephrin-A1 association, as well as subsequent signaling, might contribute to pathological misregulation of processes in breast cancer cells (Lohmüller et al., 2013). The spatial coordination of ephrin-ligand–Eph-receptor pairs on apposed cells also ensures that only receptor-bound ligands are released by the protease ADAM10, which cleaves ephrin in trans (Janes et al., 2005; Zimmer et al., 2003). Furthermore, direct regulation of receptor function by the nanoscale distribution of ligands has been shown for the ephrin-A5–EphA2 pair (Shaw et al., 2014). In this elegant work, ephrin-A5 was

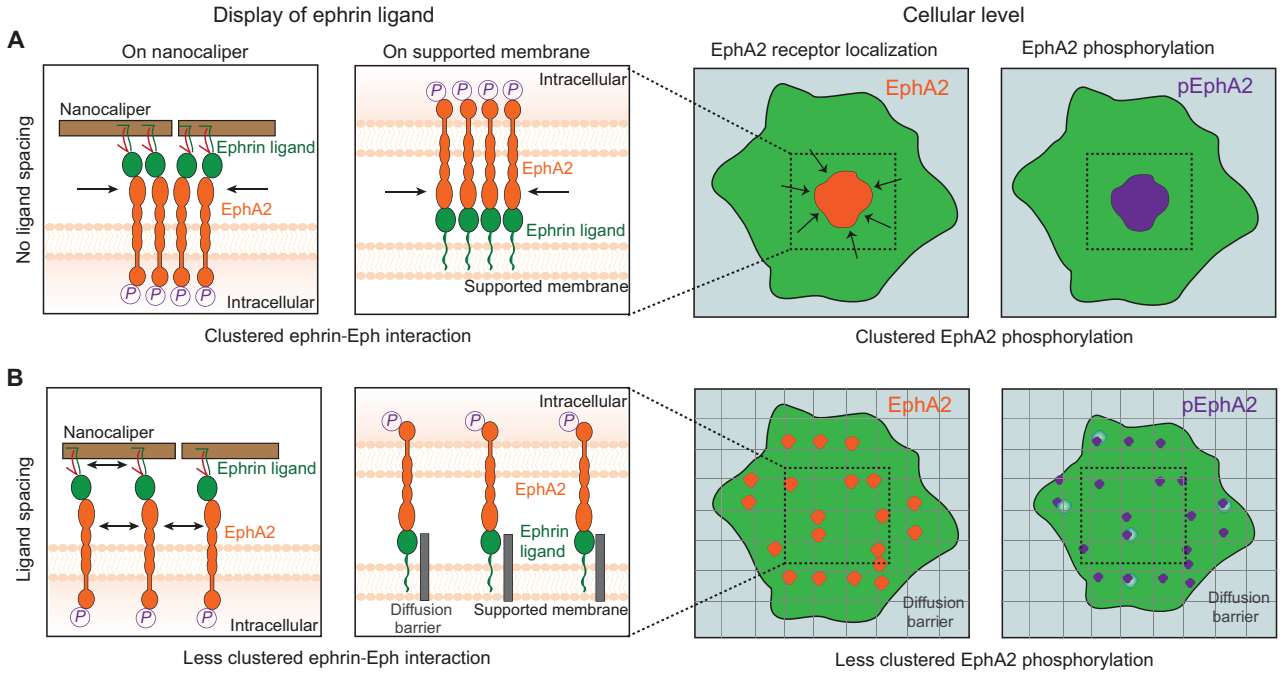

Fig. 3. Spacing of ephrin ligands defines the clustering of Eph receptors and their phosphorylation. Binding of either ephrin-A1 or ephrin-A5 to their common receptor EphA2 induces the clustering and phosphorylation of EphA2, as shown in the left panels at the level of the receptor and in the right panels at the level of the cell (Salaita et al., 2010; Shaw et al., 2014). (A) Presentation of ephrin ligands either by using DNA 'origami' nanostructures (nanocalipers) (left panel) or on supported membranes (right panel) to cells that express EphA2 receptors induces clustering and phosphorylation of the receptors at positions opposed to the ligand (in trans). (B) Increased spacing between ephrin ligands (100 nm) on nanocalipers reduces the clustering and phosphorylation of EphA2 receptors (left panel). Similarly, a nanofabricated grid positioned beneath the ephrin-presenting supported membrane can act as a physical barrier and restricts the lateral diffusion of EphA2 and its phosphorylation (right panel). The figure has been adapted from (Salaita et al., 2010) with permission. Arrows indicate the clustering or spacing of the complexes. pEphA2, phosphorylated EphA2.

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presented to EphA2-expressing cells in the form DNA 'origami' nanostructures (nanocalipers), to which the ligand has been attached at defined positions; there was either only one binding site or two binding sites separated by distances of approximately 43 or 100 nm. The resulting spatial organization of ephrin-A5 at the nanoscale determined the degree of EphA2 receptor activation depending on the ligand spacing used – ephrin-A5 dimers induced stronger receptor phosphorylation than a single ephrin-A5 molecule, and the ephrin-A5 dimer with the shorter distance between the binding sites was the most efficient ligand (Shaw et al., 2014) (Fig. 3).

The recent development of advanced microscopy techniques has allowed an increased understanding of the ligand-induced formation of receptor complexes at the nanoscale level. Observing live cells with single-molecule imaging techniques has enabled the analysis of single steps of the formation of receptor complexes from initial binding to signal transduction and amplification (Cui et al., 2007; Marchetti et al., 2013; Shibata et al., 2006; Tani et al., 2005), and this has also been studied in other cellular systems, such as in the immune system (see Box 1). For instance, the spatio-temporal mobility of fluorescently labeled NGF (NGF–Cy3) upon receptor binding has been described as comprising at least two distinct states, characterized as a mobile and immobile phase. Initially, many of the receptor-bound NGF–Cy3 molecules are mobile, and their subsequent immobilization is associated in time with intracellular signaling. Dual live-imaging has illustrated the recruitment of intracellular signaling components to the immobile NGF-receptor complexes (Shibata et al., 2006). Moreover, the differential biological effects of three different neurotrophins – NGF, NT3 and proNGF – correlate with different patterns of ligand-induced reorganization of quantum-dot-labeled TrkA – i.e. immobile versus mobile state at the cell membrane – and quantitative differences in the lateral mobility patterns and trajectories that are induced by ligand binding (Marchetti et al., 2013). Furthermore, on chicken growth cones, only 40 Cy3-conjugated NGF molecules, which bind to approximately 5% of the available high-affinity receptors, are necessary to initiate the motile responses of the growth cones (Tani et al., 2005). Upon binding of NGF–Cy3 to these receptors, the complexes are transported laterally and unidirectionally towards the central region of the growth cone. This transport is driven by actin and is a crucial intermediate step before subsequent endocytosis of the NGF-receptor complexes, which are transported to the cell body in a retrograde manner afterwards (Tani et al., 2005). For the neurite growth inhibitory membrane protein Nogo-A, an increased mobility of the Nogo-A co-receptor TSPAN-3 is observed upon ligand binding. TSPAN-3 molecules are released from areas that had originally been confined and exhibit increased lateral diffusion. Subsequently, the Nogo-A–TSPAN-3 complexes interact with the signal-transducing receptor S1PR2, forming large multi-subunit complexes (N.K.T.-S., Björn Tews, David Albrecht, Zorica Ristic, Helge Ewers and M.E.S., unpublished observation). In conclusion, these studies illustrate the sensitivity of specific attractive or repulsive receptors to the presentation of their ligand, which is reflected in changes in mobility at the nanoscale and microscale levels.

Internalization and intracellular trafficking

Experiments in the early 1970s revealed the uptake of purified radiolabeled NGF by nerve endings of sympathetic and sensory neurons, and its subsequent retrograde transport to the cell body (Angeletti et al., 1972; Hendry et al., 1974; Stoeckel et al., 1975). Since then, several groups have revealed crucial insights into the cellular mechanisms of the internalization, trafficking and retrograde transport of neurotrophic factors and pro-neurotrophins (Boutilier et al., 2008; Bronfman et al., 2003; Harrington and Ginty, 2013; Nykjaer et al., 2004; Zweifel et al., 2005). In particular, the use of fluorescent labeling techniques and live imaging have made it possible to investigate kinetic aspects, such as the time course of neurotrophin binding, ligand concentration, and the recruitment of interaction partners and accessory factors relevant for correct internalization (Bronfman et al., 2003; Gatzinsky et al., 2001; Jullien et al., 2003; Levi et al., 1980; Philippidou et al., 2011; Valdez et al., 2007; Zweifel et al., 2005). At the single-molecule level, the distinct progressive phases of NGF– or BDNF–receptor complex formation and its endocytic trafficking have been characterized in live cells by using fluorescent or quantum-dot-labeled NGF and BDNF. Such characterization at the single-molecule level further suggests that the dynamic regulation of ligand–receptor interaction controls the strength and duration of the downstream signaling (Rajan et al., 2008; Vermehren-Schmaedick et al., 2014).

Recently, the dynamic control of ligand–receptor interactions has also been shown at the level of transcytosis between different cellular compartments – NGF binding to the TrkA receptors at the growth cone induces the recruitment of additional TrkA receptors from the neuronal soma surface, which are transported to the distant growth cones. This intracellular translocation of TrkAs from regions with low levels of NGF to regions with higher concentrations of NGF enables the adjustment of the neuronal sensitivity to the presence of the ligand (Ascano et al., 2009).

In addition to neurotrophins, several other signaling complexes that mediate attractive and repulsive axonal guidance cues also undergo

Box 1. Multi-subunit receptor complex dynamics at the immunological synapse

In response to surface-bound antigens on antigen-presenting cells (APCs), B- and T-cell lymphocytes mediate adaptive immune responses through the activation of their B- and T-cell receptors (BCRs and TCRs, respectively) (Brownlie and Zamoyska, 2013; Friedl et al., 2005; Lund and Randall, 2010). Similar to the receptor complexes that are formed by attractive and repulsive nerve fiber growth regulators, TCRs and BCRs signal in concert with several co-receptors and supplementary factors, as revealed, for example, by advanced microscopy techniques (Douglass and Vale, 2005; Grakoui et al., 1999; Monks et al., 1998). Briefly, the following events occur at an immunological synapse (the interface between APCs and lymphocytes) (Douglass and Vale, 2005; Grakoui et al., 1999; Monks et al., 1998) – the recognition of antigens induces the TCR co-receptor CD4 to associate with CD3 chains, as shown by fluorescent resonance energy transfer (FRET) live imaging (Żal et al., 2002). Single-molecule microscopy has also revealed the dynamic nature of specific membrane subdomains (Douglass and Vale, 2005) – the co-receptor CD2 and the kinase Lck, which phosphorylates conserved immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3, are recruited to the activated TCR, together with the adapter protein LAT. By contrast, the phosphatase CD45 (also known as PTPRC), a potential inhibitor of TCR signaling, has been found to be excluded from the observed TCR microdomains (Douglass and Vale, 2005). Signal induction is followed by the recruitment of kinases and signaling molecules, leading to the activation of the Ras–MEK–ERK pathway and cytokine (IL-2) production, which promotes the proliferation of activated T-cells (Friedl et al., 2005).

The formation of the BCR complex and its signaling are regulated in a similar dynamic manner (Mattila et al., 2013; Monroe, 2006). Taken together, the concerted signaling that is mediated by receptor complexes on lymphocytes and their partners is organized in a spatio-temporal manner, and their misregulation might result in immune deficiencies or lymphocyte malignancies (Conley et al., 2009; Rickert, 2013; van Zelm et al., 2010).

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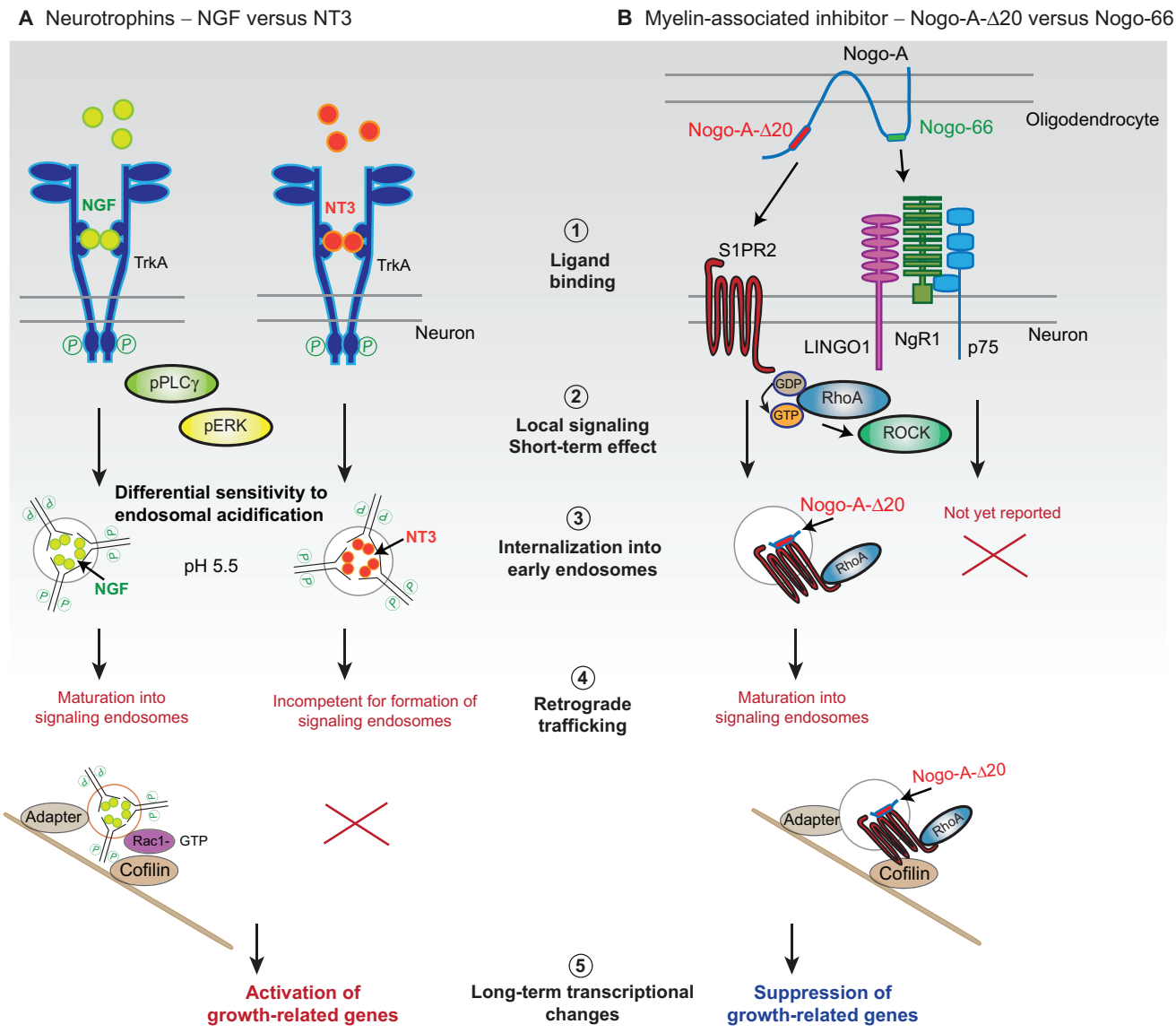

Fig. 4. Ligand-selective internalization and translocation of binding partners governs local (axonal versus global) cellular effects. Growth-promoting molecules, such as the neurotrophins NGF and NT3 (A), or growth-restricting molecules, such as the active Nogo-A domains Nogo-A-Δ20 and Nogo-66 (B) interact at the neuronal growth cone with their respective receptors. (*In vivo*, full-length Nogo-A might undergo transcytosis or proteolytic cleavage, resulting in the release of active fragments such as Nogo-A-Δ20.) Multi-subunit receptor complexes assemble and induce downstream signaling, such as the activation of GTPases (Rac1 for neurotrophins and RhoA for Nogo-A), which influences the local cytoskeleton of the growth cone (to promote outgrowth, turning or collapse). Formation of receptor complexes is often followed by internalization into early endosomes, i.e. in the case of NGF–TrkA, NT3–TrkA or Nogo-A-Δ20–S1PR2. Internalization of Nogo-66 and its receptors has not been demonstrated yet. Intracellular sorting sends the endosomes for degradation, recycling or retrograde transport to the cell soma. Nogo-A-Δ20-containing endosomes and NGF–TrkA-containing endosomes undergo retrograde trafficking, whereas NT3–TrkA-containing endosomes are unable to follow this transport route. The differential recruitment of actin modifiers (such as Rac1 and cofilin-family members) results in unstable NT3–TrkA complexes within the acidic environment of early endosomes and so restricts their signaling competence to only a short distance. By contrast, NGF and Nogo-A-Δ20 continue to signal in the cell body and thus exert a long-term effect through modulating the levels of CREB phosphorylation, which affects the subsequent expression of neurite growth-specific proteins and transcription factors. pERK, phosphorylated ERK; pPLCγ, phosphorylated PLCγ.

endocytosis. The Nogo-A inhibitory fragment Nogo-A-Δ20 is internalized at neuronal growth cones by the same pinocytic chaperone protein Pincher [also known as EHD4, Eps15 homology (EH)-domain-containing proteins], which is also important for endocytosis of the NGF–TrkA complex (Joset et al., 2010; Philippidou et al., 2011). Internalized Nogo-A-Δ20 colocalizes with its receptor S1PR2 and its co-receptor TSPAN-3 in early endosomes (Kempf et al., 2014) (N.K.T.-S., Björn Tews, David Albrecht, Zorica Ristic, Helge Ewers and M.E.S., unpublished observations). Neurite outgrowth inhibition by MAG depends on LRP1-mediated endocytosis (Stiles et al., 2013), probably together with β1-integrins (Hines et al., 2010). Ephrin-mediated repulsion is regulated by endocytosis of either full-length ligand–receptor complexes or ephrin-B ligands that have been cleaved by metalloproteinases (Marston et al., 2003; Zimmer et al., 2003). The above examples illustrate the wide-spread occurrence of the internalization of attractive and repulsive ligand–receptor complexes following initial complex assembly, but this raises the question of its functional relevance for specific intracellular signaling.

From local effects at axons to control at the cell body

The translocation of ligands, such as neurotrophins and Nogo-Δ20, and their receptors from distal axons to neuronal cell bodies allows cells to directly propagate signaling from the distal neurites to perinuclear regions (Harrington and Ginty, 2013; Joset et al., 2010; Winckler and Yap, 2011). Signaling endosomes that have been transported contain the endocytosed ligand–receptor complexes and associated downstream effectors, such as small G-proteins, Rho-GTPases, constituents of the phospholipase C-γ (PLCγ) cascade, Raf (a serine/threonine protein kinase), mitogen-activated protein kinase kinase (MEK) and phosphatidylinositol 3-kinase (PI3K) (Ginty and Segal, 2002; Harrington et al., 2011; Joset et al., 2010; Zweifel et al., 2005). Quantitative analysis revealed that endosomes that contain as little as a single NGF dimer can undergo retrograde trafficking (Cui et al., 2007). Once the signaling endosomes reach the cell body, neurotrophic factors enhance the phosphorylation or activation of the transcription factor cyclic AMP response element-binding protein (CREB) and the expression of neurite growth-specific proteins and transcription factors, whereas Nogo-A-Δ20 exerts an opposite growth-inhibitory effect (Ginty and Segal, 2002; Harrington and Ginty, 2013; Montani et al., 2009; Riccio et al., 1997; Schwab, 2010) (Fig. 4). Long-term inhibition of neurite outgrowth by Nogo-Δ20–S1PR2–TSPAN-3 signaling is probably linked to cell-body-mediated effects through signaling endosomes that have undergone retrograde transport, whereas, Nogo-66- or MAG-induced growth-cone collapse is triggered locally by the activation of the RhoA–ROCK axis, which leads to destabilization of the cytoskeleton in a manner that is independent of protein synthesis (Chivatakarn et al., 2007; Joset et al., 2010; Kempf et al., 2014; Manns et al., 2014) (Fig. 4).

A local short-term effect at the axon versus a central effect at the cell body for long-term control of neurite outgrowth has also been described for the activation of TrkA by NT3 and NGF, respectively. TrkA signaling that is induced by NT3 elicits local signaling that is important for the finding of intermediate targets (e.g. axon extension along the vasculature) (Ascano et al., 2012; Bodmer et al., 2011). By contrast, TrkA signaling that is induced by NGF results in its retrograde transport and the activation of calcineurin (a phosphatase that dephosphorylates the transcription factor NFAT), leading to NFAT-mediated transcriptional control of growth-promoting genes (Ascano et al., 2012; Bodmer et al., 2011). Interestingly, a proteomic approach (Harrington et al., 2011) has identified the differential recruitment of actin modifiers to endosomes containing either NGF–TrkA or NT3–TrkA; the association of Rac1 and cofilin proteins, which occurs exclusively at NGF–TrkA-containing endosomes, is essential for the maturation of early-to-late endosomes that are competent for retrograde transport. The inability of NT3–TrkA-containing endosomes to associate with and to activate the actin modifiers Rac1 and cofilin-family members leads to unstable NT3–TrkA complexes within the acidic environment of the early endosomes and to the restriction of their signaling to only short-term local effects (Harrington et al., 2011). Thus, it is clear that the differential sorting of receptor complexes and their binding partners following internalization promotes spatio-temporal fine-tuning of signaling events.

Conclusions

Many important events controlling the growth, plasticity and regeneration of nerve fibers are regulated by growth factors, growth inhibitors and attractive or repulsive guidance factors, which act as ligands to activate specific receptors on the membrane of the responsive cells and the neuritic growth cones. The binding of these ligands induces the recruitment of additional interaction partners in the cell membrane, leading to heteromeric multi-subunit receptor complexes. These processes modify the strength, duration and subcellular localization of the induced downstream signaling. Single ligand–receptor dimers can be sufficient for potent signal transduction, but microdomains comprising multimerized complexes that contain multiple ligands, receptors, adaptors, modifiers and scaffolding components seem to be a common occurrence. The quantitative composition of these ligand–receptor clusters can determine the strength of the cellular responses that are elicited.

Future microscopy and biochemical techniques using higher and more sensitive resolution will help to refine our current knowledge of the dynamics of receptor complexes, and the signaling mechanisms of growth regulators and attractive and repulsive axonal guidance cues, which affect the development, health and disease of the nervous system.

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Competing interests

The authors declare no competing or financial interests.

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